ELSEVIER

Antiviral Research 29 (1996) 243–260

Antiviral Research

Antisense oligodeoxynucleotides against the BZLF1 transcript inhibit induction of productive Epstein-Barr virus replication

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Received 5 May 1995; accepted 12 October 1995

Abstract

Expression of the Epstein-Barr virus (EBV) BZLF1 gene product, ZEBRA, in latently infected cells is sufficient to induce the viral lytic cycle. The use of oligodeoxynucleotides complementary to the BZLF1 transcript was studied to inhibit this induction of productive viral replication. For this purpose, we employed oligodeoxynucleotides complementary to the translation initiation codons and their flanking sequences. Incubation of Akata cells with the 25-mer phosphodiester (PO)- or phosphorothioate (PS)-antisense oligodeoxynucleotides for 3 h before stimulation with anti-immunoglobulin G antibodies (anti-IgG) partially inhibited the anti-IgG-mediated induction of ZEBRA synthesis. Both the PO- and PS-antisense oligodeoxynucleotide treatments also suppressed the productive EBV replication (as measured by linear DNA production) in a dose-dependent manner, with much greater efficiency than did PO- and PS-oligodeoxynucleotides with sense, reverse or random sequences of the same length. Another 20-mer antisense oligodeoxynucleotide complementary to sequences downstream of the translation initiation codons showed a similar inhibitory effect on EBV replication. However, the inhibition was considerably lower when the cells were treated with oligodeoxynucleotides complementary to sequences upstream of the start codons. These results indicate that BZLF1 antisense oligodeoxynucleotides inhibit the viral activation in a sequence-specific fashion. In the virus-producer cell line P3HR-1, the same PS-antisense oligodeoxynucleotides also partially suppressed the spontaneous viral replication after 6–10 days, substantially more than the PS-random oligodeoxynucleotides. Inhibition of BZLF1 appears to be sufficient to suppress the induction of EBV replication.

Keywords: Epstein-Barr virus; Latency; Reactivation; BZLF1; Antisense; Phosphorothioates

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SSDI 0166-3542(95)00902-X

1. Introduction

Epstein-Barr virus (EBV) is a ubiquitous human herpesvirus which infects B lymphocytes and certain epithelial cells. A common property of this virus is establishment of latency following primary infection. EBV has the potential of reactivation after prolonged persistence, and is implicated in several human malignancies, including Burkitt's lymphoma (BL), nasopharyngeal carcinoma, non-Hodgkin's lymphoma, especially in immunosuppressed individuals, and probably Hodgkin's lymphoma (reviewed in Miller, 1990). EBV is also the causative agent of infectious mononucleosis.

EBV immortalizes human and non-human primate B lymphocytes in culture. In these cells, the viral genome is maintained in a plasmid form and only a limited number of viral genes is expressed (reviewed in Kieff and Liebowitz, 1990). The shift from latency to a lytic replication cycle can be induced by various reagents, such as 12-0-tetradecanoylphorbol-13-acetate (TPA) (zur Hausen et al., 1978), calcium ionophore (Faggioni et al., 1986) and anti-immunoglobulin antibody (anti-Ig) (Tovey et al., 1978; Takada and Ono, 1989; Daibata et al., 1990). These inducers act through certain signal transduction pathways which lead directly to the activation of the promoter for BZLF1 (Flemington and Speck, 1990; Shimizu and Takada, 1993; Daibata et al., 1994), an immediate early gene required for EBV activation (Countryman and Miller, 1985; Chevallier-Greco et al., 1986; Takada et al., 1986; Countryman et al., 1987). In addition to the BZLF1 gene, several other *trans*-activating EBV genes are involved in the activation of the lytic cycle. It is thought that the BZLF1, BRLF1, and BMLF1 genes together play an important role in activating further stages of the lytic cycle cascade (Lieberman et al., 1986; Hardwick et al., 1988; Kenney et al., 1989; Sinclair et al., 1991). However, several studies have shown that expression of the BZLF1 gene is the first step in this cascade, since only the product of the BZLF1 gene, ZEBRA (also referred to as Zta and EB1), is able to induce the lytic cycle when transcriptionally active gene constructs are transfected into latently infected BL cells (Countryman and Miller, 1985; Chevallier-Greco et al., 1986; Takada et al., 1986; Countryman et al., 1987). A number of cellular factors also interact with the viral genes in initiating or regulating the reactivation of the latent infection (Zalani et al., 1992; Miller et al., 1993; Sista et al., 1993; Daibata et al., 1994).

Many potential antiviral compounds have been tested for anti-EBV activity in vivo and in vitro. Some of them have been found to inhibit EBV DNA polymerase activity without affecting the expression of early antigens. These include phosphonoacetic acid (Summers and Klein, 1976), arabinofuranosylthymine (Ooka and Calender, 1980), and acyclovir (Colby et al., 1980; Datta et al., 1980). Another approach to inhibition of viral reactivation is the use of agents which specifically block the expression of a gene engaged in a critical role in the viral activation cascade. In this context, BZLF1 seems to be an ideal target to interfere with EBV replication.

Antisense oligodeoxynucleotides have been utilized as tools for inhibiting gene expression and replication of several viruses, including human immunodeficiency virus (Zamecnik et al., 1986; Matsukura et al., 1987; Goodchild et al., 1988; Agrawal et al., 1989), influenza virus (Zerial et al., 1987; Leiter et al., 1990), papillomavirus (Cowsert et al., 1993), herpes simplex virus type 1 (HSV-1) (Smith et al., 1986), human cytomegalovirus (Smith and Pari, 1995; Pari et al., 1995), and the EBNA-1 gene of EBV (Pagano et al., 1992; Roth et al., 1994). Antisense oligonucleotides can bind to the target mRNA and thereby block its expression or function (Stein and Cohen, 1988; Hélène and Toulmé, 1990). Because of the relatively short half-lives of unmodified phosphodiester (PO)-oligonucleotides in serum and in cells due to the presence of nucleases (Wickstrom, 1986), several modifications of oligonucleotides have been evaluated (Stein and Cohen, 1988). The most widely used is the phosphorothioate (PS) (Stein and Cohen, 1988). Phosphorothioates are highly resistant to nucleases, and their physicochemical properties (uptake into cells, transport into nucleus, hybridization with target mRNA, etc.) are sufficiently comparable to PO-oligonucleotides to warrant their substitution (Stein and Cohen, 1988; Stein et al., 1988).

In the present investigation, we used both PO- and PS-oligodeoxynucleotides complementary to sequences near the AUG AUG translation initiation codons of the EBV BZLF1 transcript to examine whether these antisense oligonucleotides could inhibit viral reactivation. We employed a Burkitt's lymphoma cell line, Akata, in which the viral lytic cycle can be synchronously induced in the majority of cells by treatment with anti-IgG (Takada and Ono, 1989; Daibata et al., 1990; Mellinghoff et al., 1991), and found that both the PO- and PS-antisense oligonucleotides inhibit the induction of ZEBRA synthesis and subsequent productive EBV replication. The inhibition of this induced EBV replication was oligodeoxynucleotide dependent and was EBV sequence specific. We also demonstrated similar inhibition of EBV replication by the antisense oligonucleotides in the virus-producer cell line P3HR-1.

2. Materials and methods

2.1. Cell lines

Akata (Takada and Ono, 1989), an EBV-positive Burkitt's lymphoma cell line, and P3HR-1 (Hinuma et al., 1967), an EBV-producer cell line, were maintained in RPMI-1640 medium supplemented with penicillin (100 IU/ml), streptomycin (100 μg/ml), and 10% heat-inactivated fetal calf serum in a humidified atmosphere of 5% $C0_2$ in air. Akata and P3HR-1 cells were incubated at 37°C and 33°C, respectively; the production of P3HR-1 EBV is higher upon incubation at 33°C than 37°C (Hinuma et al., 1967). The EBV lytic cycle was induced in Akata cells by treatment with 100 μg/ml of anti-human IgG (Cappel, West Chester, PA) (Daibata et al., 1990).

2.2. Oligodeoxynucleotides

PO- and PS-oligodeoxynucleotides were synthesized on an automated DNA synthesizer (Applied BioSystems Inc., Foster, CA) according to the manufacturer's instructions, purified by reverse-phase high performance liquid chromatography, detritylated, ethanol precipitated, and analyzed by polyacrylamide gel electrophoresis (PAGE). A 25-mer oligonucleotide 5'-TT TGG GTC CAT CAT CTT CAG CAA AG-3', designated Z-1, was used as an anti-BZLF1 antisense oligonucleotide (see Fig. 1). This sequence flanks the translation initiation codons (AUG AUG, underlined) of the BZLF1 transcript. Two other 20-mer anti-BZLF1 antisense oligonucleotides were also tested: 5'-CAT CAT CTT CAG CAA AGA TA-3' (Z-2 oligonucleotide) and 5'-TCA GAA GTC GAG TTT GGG TC-3' (Z-3 oligonucleotide). They are immediately adjacent to each other and partially overlap with the Z-1 oligonucleotide (see Fig. 1). The Z-2 oligonucleotide is complementary to the sequence starting within the 5' untranslated region and ending with the two copies of AUG with which the translation starts. The Z-3 oligonucleotide is complementary to a region immediately downstream of the BZLF1 translation initiation codons. Twenty-five-mer PO- and PS-oligonucleotides with random sequences (random oligonucleotides) were employed as negative controls of the antisense oligonucleotides. At each step of the synthesis of the random oligonucleotides, the synthesizer was given the free choice of all four deoxynucleotides, resulting in a random mixture of 4^{25} sequences. Similarly, two other 25-mer PS-oligonucleotides were used as negative controls; one has the complementary sequence of Z-1 (i.e. the sense sequence: 5'-CT TTG CTG AAG ATG ATG GAC CCA AA-3'); the other one is the reverse sequence of Z-1: (5'-GA AAC GAC TTC TAC TAC CTG GGT TT-3') i.e. it has the identical nucleotide composition as Z-1. All oligonucleotides were dissolved in water and stored at −20°C. The solvent itself had no inhibitory effect on induction of EBV antigens and linear EBV DNA (data not shown).

2.3. Oligonucleotide treatments of cells

Logarithmically growing Akata cells were resuspended to a final concentration of 1×10^6 cells/ml in fresh medium. The cells were incubated in the presence of various concentrations of PS- or PO-oligonucleotides for 3 h at 37°C, and then stimulated with anti-human IgG for 24 h in the continued presence of the oligonucleotides.

For the treatment of P3HR-1 cells with the PS-oligonucleotides, the cells (1×10^6 cells/ml) received the oligonucleotides at the indicated concentrations, and the incubations were continued for 16 days at 33°C. Every second day, the cell concentration was adjusted to 1×10^6 cells/ml by adding fresh medium containing the original concentration of PS-oligonucleotides. Aliquots were taken for EBV DNA analysis and immunofluorescence at several time points.

2.4. EBV DNA analysis (Gardella gel analysis)

The quantity of linear EBV DNA was determined by gel electrophoresis followed by hybridization of the transferred gels (Gardella et al., 1984). This technique allows the resolution of linear and covalently closed, circular (CCC) viral DNA in EBV-infected cells. Briefly, 0.5×10^6 Akata cells or 1.0×10^6 P3HR-1 cells were suspended in 100 μl of sample buffer containing 15% Ficoll and 0.01% bromophenol blue in TBE buffer (89 mM Tris, 89 mM boric acid, 2.5 mM EDTA, pH 8.3) and loaded into the well of a vertical 0.75% agarose gel. Lysis buffer (100 μl), containing 5% Ficoll, 1% sodium dodecyl sulfate (SDS), 1.5 mg/ml pronase, and 0.05% xylene cyanol green in TBE buffer, was layered over the samples. Electrophoresis was carried out at 20 V for 3 h and then at 80 V for 14 h at 4°C. The DNA in the gel was partially depurinated with 0.25 M HCl, denatured with 0.4 M NaOH in 0.5 M NaCl, and transferred to nylon-membrane filters (MagnaCharge; Micron Separation Inc., Westboro, MA) using downward capillary alkaline transfer. The papers were baked at 80°C for 1 h, treated with prehybridization buffer, and hybridized with a random-primed ^{32}P-labeled EBV *Bam*HI-W probe. After washing, the filters were exposed to Kodak XAR films at −80°C with intensifying screens. The amount of linear EBV DNA was quantitated with a bioimage analyzer (Betagen; IntelliGenetics Inc., Mountain View, CA).

To quantitate the amounts of EBV DNA which remains with the cell DNA on top of the gel ('well DNA') (Severini et al., 1994) the amounts of cells were reduced to 0.1×10^6 cells per well. The cells were suspended in 50 μl 2 × concentrated modified sample buffer A (containing 4% Ficoll instead of 20%, after mixing with agarose) at 37°C and mixed with 50 μl 1.5% low melting point agarose (Sea Plaque, FMC Bioproducts, Rockland, ME) at 37°C and immediately loaded in the well of the gel. Lysis buffer B (100 μl) was added after the cell suspension in agarose had solidified. Transfer of the DNA and hybridization were performed as described above.

2.5. Immunofluorescence

Indirect immunofluorescence was used for staining of diffuse early antigen (EA-D) and viral capsid antigen (VCA). EA-D, the BMRF1 gene product, was detected with murine IgG_1 monoclonal antibody (mAb) 9240 (NEN/DuPont, Wilmington, DE), which is identical to the R3 mAb (Pearson et al., 1983). This mAb recognizes the EA-D complex at 50–55 kDa in EBV-activated B cells (Pearson et al., 1983; Daibata and Sairenji, 1993). VCA was detected with a seropositive human serum (anti-VCA 1:160; anti-EA less than 1:10) from a healthy donor (Daibata et al., 1990). Cells were washed with phosphate-buffered saline (PBS) and spotted on a glass slide, dried, and fixed in acetone at −20°C for 15 min. The fixed smears were incubated with the first antibody at 37°C for 40 min. After washing in PBS, the slides were incubated at 37°C for 40 min with the fluorescein isothiocyanate-conjugated anti-mouse or anti-human IgG (Cappel) corresponding to the first antibody. The slides were washed and mounted in 1:1 glycerol/PBS, and examined under a fluorescence microscope. At least 500 cells were counted for each determination.

2.6. Western blot analysis

ZEBRA was detected by Western immunoblotting using a rabbit polyclonal antibody, a gift from G. Miller (Taylor et al., 1989). This antibody recognizes a 38-kDa ZEBRA protein in Akata cells after cross-linking with anti-IgG (Daibata et al., 1991, 1992). Cells were washed

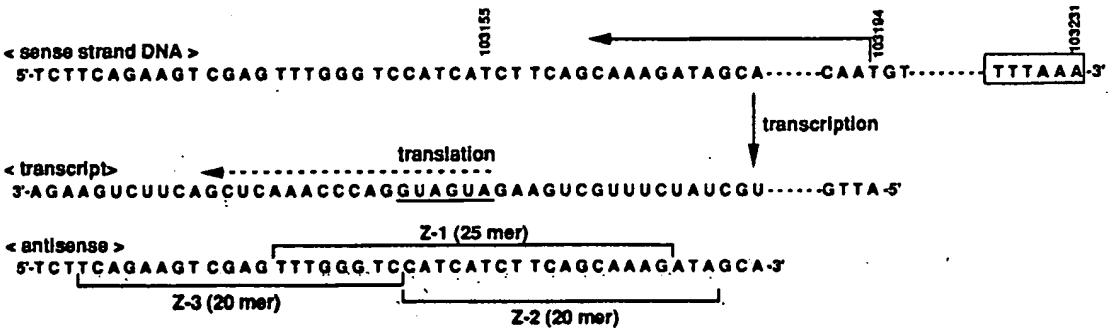

Fig. 1. Location and sequences of antisense oligodeoxynucleotides against the BZLF1 transcript (Z-1, Z-2 and Z-3) used to inhibit the EBV lytic cycle. Nucleotide sequences of the sense strand DNA and of the leftward transcript of BZLF1 of Akata, P3HR-1 and B95-8 viruses are shown (Biggin et al., 1987; Jenson and Miller, 1988; Packham et al., 1993). The leftward arrow with a solid line indicates the site of transcription initiation. The initiation site of translation is underlined. The TATA box (AAATTT) in the BZLF1 promoter is boxed. The numbers represent nucleotide numbers on the B95-8 EBV DNA map (Baer et al., 1984).

with PBS and resuspended in lysis buffer containing 10 mM Tris–HCl (pH 7.2), 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, 2 mM phenylmethylsulfonyl fluoride and 1 μg/ml aprotinin for 20 min on ice. After sonication, the lysates were centrifuged at 15000 × g at 4°C and the supernatants were collected. Each sample was diluted with an equal volume of 2 × SDS sample buffer (125 mM Tris–HCl, pH 6.8, 4.6% SDS, 10% 2-mercaptoethanol, 20% glycerol), heated at 100°C for 5 min, and subjected to 10% SDS–PAGE. The proteins were electrophoretically transferred onto nitrocellulose filters (Hybond-C; Amersham, Arlington Height, IL) in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol, pH 8.3) at 100 V constant voltage for 2 h. Residual binding sites on the nitrocellulose filters were blocked for 1 h with Tris-buffered saline (20 mM Tris–HCl, pH 7.5, 500 mM NaCl) containing 3% gelatin. The filter was incubated overnight with a 1:200 dilution of the antibody to ZEBRA in Tris-buffered saline plus 0.05% Tween 20 in the presence of 1% gelatin followed by incubation with ^{125}I-protein A (NEN/DuPont). The filters were exposed to Kodak XAR films. The amount of ZEBRA was quantitated with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

3. Results

3.1. Effect of anti-BZLF1 antisense oligonucleotides on the expression of EBV antigens in the viral lytic cycle

We employed PS- and PO-oligonucleotides complementary to sequences of the BZLF1 gene transcript flanking the translation initiation codons (Z-1 oligonucleotides) (Fig. 1) to test whether the antisense oligonucleotides inhibit ZEBRA synthesis. Akata cells were incubated with the antisense oligonucleotides or with random oligonucleotides for 3 h, stimulated with anti-IgG, and the cell lysates were analyzed for ZEBRA expression by immunoblotting with anti-ZEBRA antibody (Fig. 2). ZEBRA expression was undetectable in unstimulated cells. After stimulation with anti-IgG, ZEBRA was abundantly detected as a single band at 38 kDa, as expected. ZEBRA expression was suppressed by 70–90% when the cells were treated with the PS-antisense oligonucleotide at 2.5–12.5 μM. Likewise, the PO-antisense oligonucleotide inhibited ZEBRA expression, but the effect was not as strong as with the PS-antisense oligonucleotide. Only a low inhibition was observed in the cells treated with the same concentration of random oligonucleotides of the same length and nucleotide modification. (As shown in Fig. 2, the polyclonal antibody,

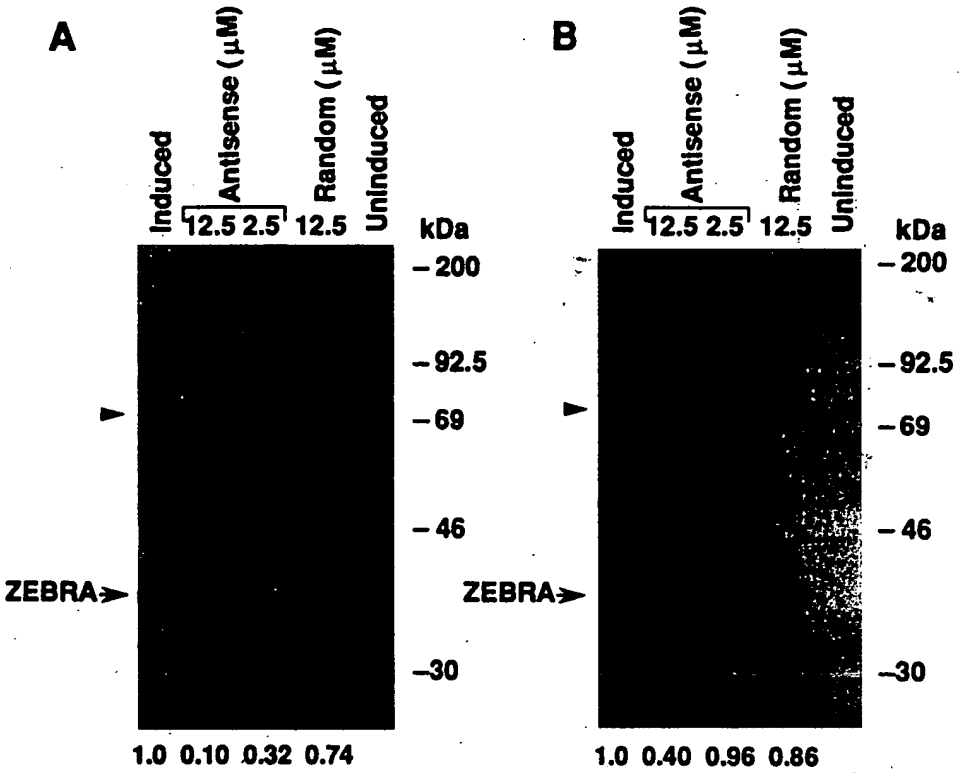

Fig. 2. Immunoblot analysis of ZEBRA induction in antisense oligonucleotide-treated cells. Akata cells were incubated for 3 h with the anti-BZLF1 antisense oligonucleotide Z-1 or with the random oligonucleotides, and then stimulated with anti-IgG for 24 h. The proteins in the cell extracts were separated by SDS–PAGE and electrotransferred to nitrocellulose filters. The filters were reacted with an anti-ZEBRA antibody followed by incubation with ^{125}I -protein A. Two control samples without the oligonucleotide treatments are included (induced, anti-Ig-stimulated cells; uninduced, unstimulated cells). The level of ZEBRA expression, normalized to the content of 72-kDa protein (arrowhead), is shown below each lane as relative amount compared with that from anti-Ig-stimulated cells without the oligonucleotide treatment. Positions of weight markers are indicated at right. (A) PS-oligonucleotides; (B) PO-oligonucleotides.

also recognized some other proteins which are presumably cellular or latent viral proteins, since their intensities were comparable before and after anti-IgG stimulation and with or without oligonucleotide treatment.) To exclude that the suppression of ZEBRA expression by Z-1 oligonucleotides was merely the result of a general suppression of protein synthesis, we determined the expression of the B-cell membrane protein CD 19 in the presence or absence of the Z-1 or the random PS-oligonucleotides. CD 19 is a membrane protein with a half life of 14–16 h (Matsumoto et al., 1991); its expression was determined by FACS analysis using the anti-CD 19 monoclonal antibody, clone HD 37. The PS-oligonucleotides had no effect on CD 19 expression either before or after anti-IgG stimulation of Akata cells (not shown).

The effect of anti-BZLF1 antisense oligonucleotides on the induction of EA-D and VCA in anti-IgG-stimulated Akata cells was assessed by immunofluorescence (Fig. 3). Only 0.3% of the Akata cells were EA-D- and VCA-positive before stimulation with anti-IgG. EA-D and VCA appeared in about 40% and 30% of the cells, respectively, at 24 h after anti-IgG stimulation. Both the

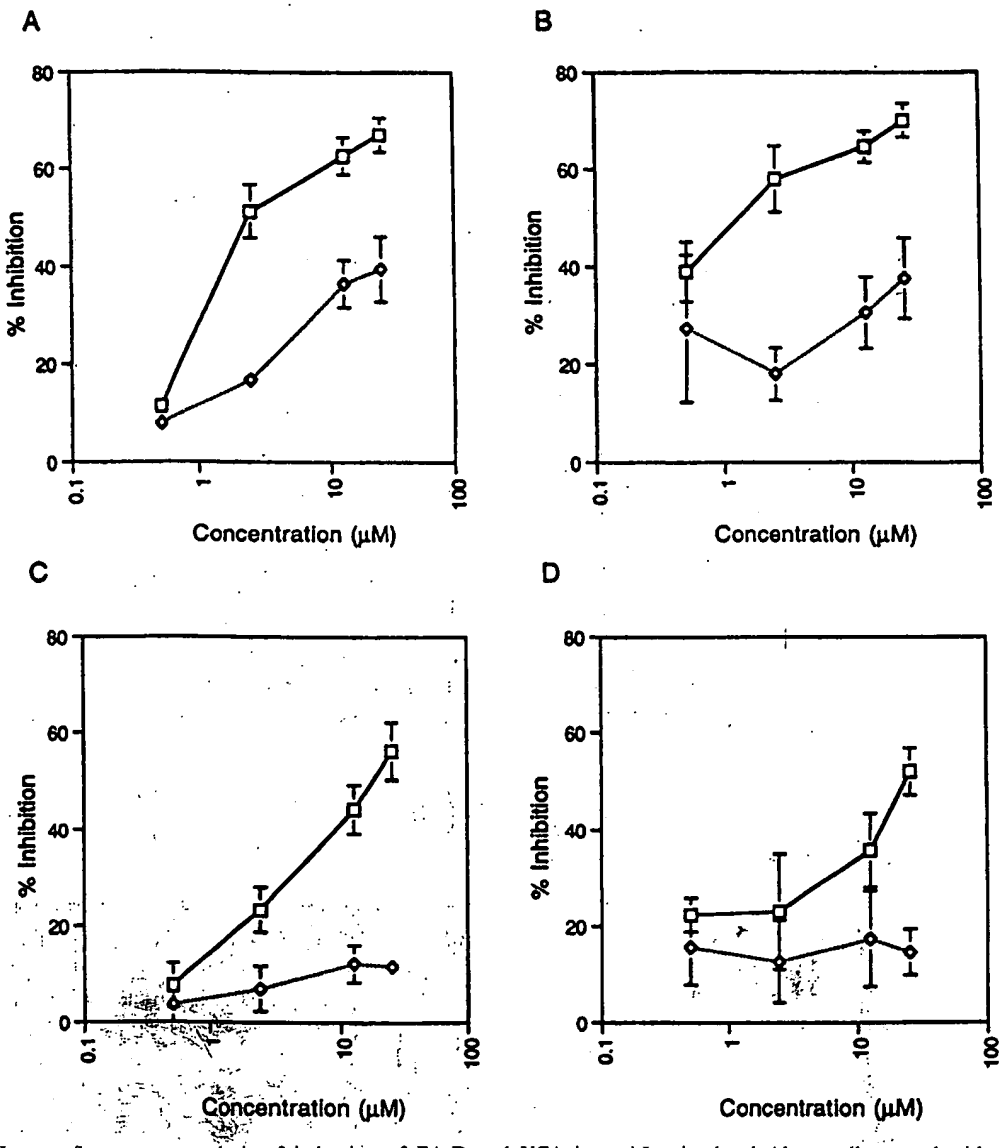

Fig. 3. Immunofluorescence analysis of induction of EA-D and VCA in anti-Ig-stimulated Akata cells treated with antisense oligonucleotides. Akata cells were incubated for 3 h with various concentrations of the PS- or PO-antisense oligonucleotide (Z-1) or with the PS- or PO-random oligonucleotides, before stimulation with anti-IgG. EA-D and VCA induction was assayed at 24 h after anti-IgG stimulation. (A and C), EA-D expression; (B and D), VCA expression; (A and B), PS-oligonucleotides; (C and D), PO-oligonucleotides. The percent inhibition is calculated for each experiment as 1 − (% of cells fluorescence positive in absence of oligonucleotides) ÷ (% of cells positive in presence of oligonucleotides). The % of Akata cells induced in the absence of oligonucleotides varied from 32% to 65% in the seven experiments depicted here. ——□——, PS antisense; - - -◇- - -, PS random; ——■——, PO antisense; - - -◆- - -, PO random.

PO- and PS-antisense oligonucleotides suppressed the EA-D and VCA induction in a concentration-dependent manner. At 25 μM PS- and PO-oligonucleotides, the highest concentration tested,

Table 1
Inhibition of productive EBV replication by anti-BZLF1 antisense oligonucleotides in anti-IgG-stimulated Akata cells[a]

Oligonucleotides	Mean ± S.E. (% inhibition)			
	0.5 μM	2.5 μM	12.5 μM	25.0 μM
Phosphorothioate				
Antisense	34 ± 6 (3)[b]	48 ± 13 (3)	68 ± 9 (4)	69 ± 5 (4)
Random	5 ± 5 (3)	21 ± 10 (3)	24 ± 9 (4)	32 ± 10 (4)
Sense[c]			11 (1)	36 (1)
Reverse[d]			17 (1)	36 (1)
Phosphodiester				
Antisense	16 ± 13 (3)	25 ± 13 (3)	46 ± 10 (3)	56 ± 8 (3)
Random	14 ± 14 (3)	14 ± 7 (3)	21 ± 3 (3)	19 ± 6 (3)

[a]Oligonucleotide-treated Akata cells were stimulated with anti-IgG for 24 h. Inhibition was measured by the amounts of linear EBV DNA from Gardella gel analysis.
[b]The number of experiments (*n*) is listed in parentheses.
[c]Sense is the sequence complementary to the Z-1 antisense.
[d]Reverse is the sequence with the identical nucleotide sequence as Z-1 but read in the opposite direction.

the number of EA-D- and VCA-positive cells was reduced by about 70% and 50%, respectively. Although the PS-random oligonucleotides also showed an inhibition of the expression of these antigens, this reduction was substantially less than the sequence-specific PS-antisense compound. The PO-random oligonucleotides did not significantly decrease the number of EA-D- and VCA-positive cells. These results were also confirmed by FACS analysis with an anti EA-D monoclonal antibody (E.-M. Enzinger and C. Mulder, unpublished data).

3.2. Anti-BZLF1 antisense oligonucleotides inhibit induction of productive EBV replication

In order to test the effects of anti-BZLF1 antisense oligonucleotides on the replication of EBV, Akata cells were stimulated with anti-IgG in the presence or absence of the Z-1 antisense oligonucleotides or of the random oligonucleotides for 24 h, and the amounts of linear EBV DNA were quantified by the Gardella gel technique (Table 1). Linear DNA is the structure found in virions and is associated with productive EBV replication. Representative results from several independent experiments are shown in Fig. 4. The levels of linear EBV DNA in uninduced cells were low, but the amounts increased dramatically after stimulation of the Akata cells with anti-IgG. In the cells pretreated for 3 h with the antisense oligonucleotides, there was a reduction in amounts of linear EBV DNA which was dose-dependent over a 2.5–25 μM range of the antisense oligonucleotides. The PS- and PO-oligonucleotides, in a range of 12.5–25 μM, blocked the induction of linear EBV DNA by approximately 70% and 50%, respectively (Fig. 4 and Table 1). A reduction in the amounts of linear EBV DNA was also observed in the cells treated with the PS-random or the sense or the reverse sequence oligonucleotides, but this reduction was substantially less than that in antisense oligonucleotide-treated cells (Table 1). The PO-antisense oligonucleotide had a similar effect as the PS-antisense oligonucleotide, but at a 10-fold higher concentration. Concomitant with the reduction of linear EBV DNA, the amounts of circular EBV DNA slightly decreased in the cells treated with antisense oligonucleotides. As shown in Fig. 4, induction of the lytic cycle in Akata cells also results in a dramatic increase in EBV-specific DNA on top of the gel. This DNA which is unable to enter the gel (similar to high molecular weight cellular DNA), probably represents replicating EBV DNA, both rolling circle and branched DNA as was shown for HSV-1 by Severini et al. (1994) and, to a lesser extent,

nicked circular and replicating episomal EBV DNA. In separate experiments and using a modification of the Gardella gel technique (see Materials and methods), it was shown that this 'well

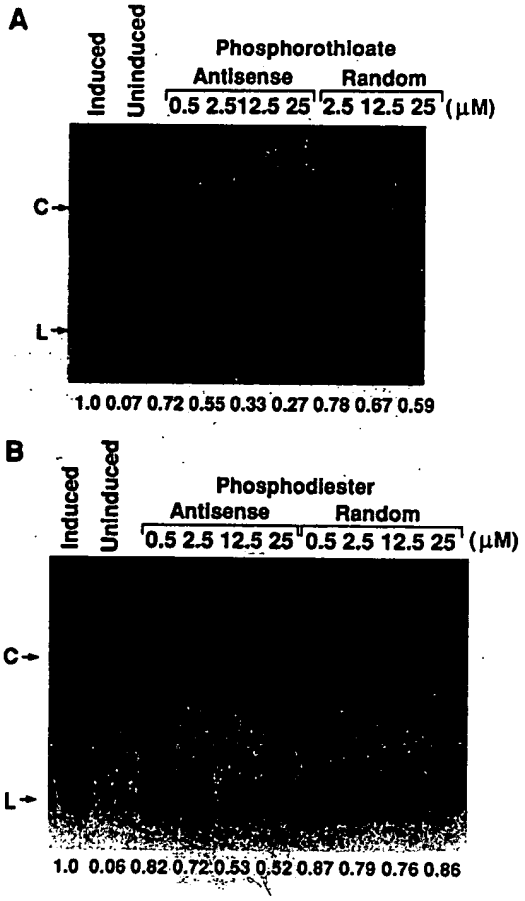

Fig. 4. EBV DNA replication analyzed by the Gardella gel technique in antisense oligonucleotide-treated Akata cells. Akata cells were incubated for 3 h with various concentrations of the PS- or PO-antisense oligonucleotide Z-1, or with the PS- or PO-random oligonucleotides before stimulation with anti-IgG and analyzed 24 h later. The cells (5×10^5) were loaded in the wells of an agarose gel, lysed, subjected to electrophoresis, and transferred to nylon-membranes for EBV DNA hybridization. Two control samples without the oligonucleotide treatments are included (induced, anti-Ig-stimulated cells; uninduced, unstimulated cells). The concentration of linear EBV DNA relative to anti-Ig-stimulated cells without the oligonucleotide treatment is indicated below each lane. (A) PS-oligonucleotides; (B) PO-oligonucleotides. C, circular EBV DNA; L, linear EBV DNA.

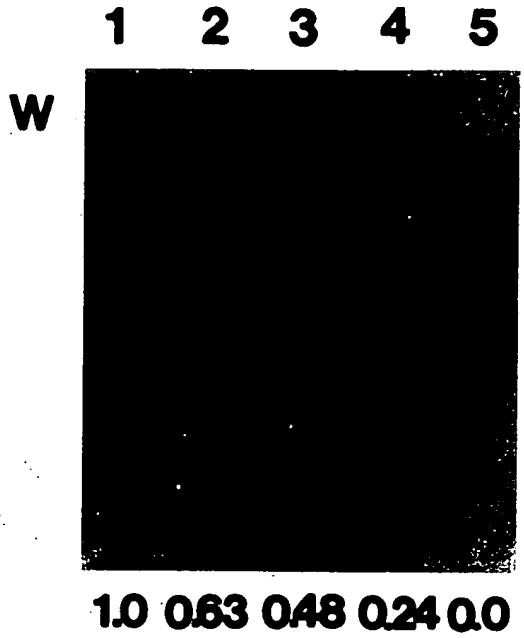

Fig. 5. Well DNA is proportional to the amount of Akata cells. Akata cells were induced with anti-IgG for 20 h. Various amounts of Akata cells were mixed with (EBV-negative) CEM T-cells to a total amount of 0.1×10^6, centrifuged, resuspended in 50 μl modified sample buffer A at 37°C, mixed with 50 μl 1.5% low melting point agarose at 37°C and loaded in the wells of a regular Gardella gel. After solidifying, the wells were overlayed with 100 μl lysis buffer B. Electrophoresis and transfer were done as before (see Fig. 4). The amounts of 'well DNA' relative to that of undiluted induced Akata cells (lane 1) is shown below each lane. The amounts of Akata cells (and CEM cells) in each lane is: lane 1; 1×10^5 (0); lane 2, 0.75×10^5 ($+0.25 \times 10^5$); lane 3, 0.5×10^5 ($+0.5 \times 10^5$); lane 4, 0.25×10^5 ($+0.75 \times 10^5$); lane 5, 0($+1 \times 10^5$).

DNA' is decreased proportional to the number of induced Akata cells (Fig. 5). For these experiments, the number of cells in each well had to be reduced 5- to 10-fold to enable a more accurate assessment of the amount of 'well DNA'. This low amount of cells does not show linear DNA in the gel (Fig. 5). Therefore, two gels had to be run, one with the low amounts of cells as in Fig. 5 for the 'well DNA' and one with the normal amount of cells ($0.5–1 \times 10^6$) for measuring the linear and the CCC DNA (not shown).

To exclude the possibility that the anti-BZLF1 antisense oligonucleotide inhibition of the produc-

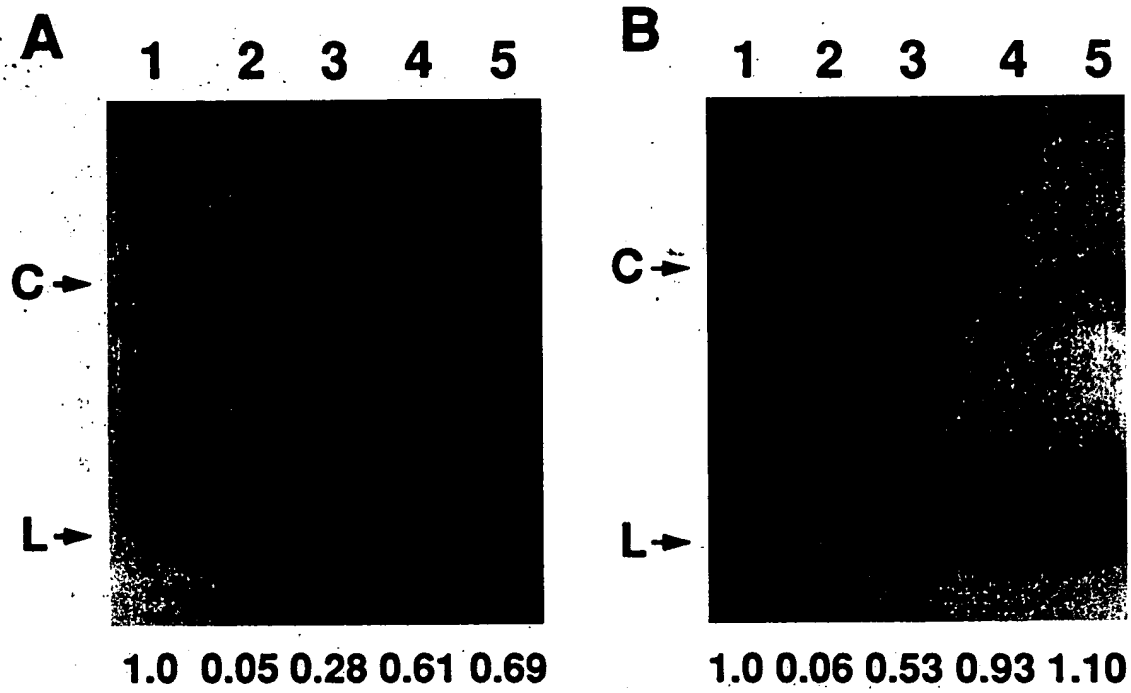

Fig. 6. Timing of antisense oligonucleotide treatment for efficient inhibition of induction of EBV replication. Antisense oligonucleotides (Z-1) were added to the cultures at various times and linear EBV DNA was analyzed by Gardella gels after 24 h incubation. The ratio of the amount of linear DNA to that in the anti-Ig-stimulated cells without the oligonucleotide treatment are shown below each lane as described in the legend to Fig. 4. Two control samples without the oligonucleotide treatments are included (lane 1, anti-Ig stimulated cells; lane 2, unstimulated cells). Antisense oligonucleotides were added: lane 3, 3 h before anti-Ig-stimulation of the cells; lane 4, simultaneously with anti-Ig; lane 5, 3 h after the stimulation. (A) PS-oligonucleotides (12.5 μM); (B) PO-oligonucleotides (25 μM). C, circular EBV DNA; L, linear EBV DNA.

tive replication of EBV was merely caused by cytotoxicity of the cells, we examined the incorporation of [^{3}H]thymidine into the cells treated with the PS-antisense oligonucleotide before and after stimulation with anti-IgG. There were no significant decreases in the levels of thymidine incorporation in antisense-treated cells even at concentration as high as 62.5 μM (data not shown). We concluded that antisense oligonucleotides against the BZLF1 transcript inhibited induction of productive EBV replication.

3.3. Pretreatment of cells is required for efficient antisense oligonucleotide inhibition of the induction of productive EBV replication

The inhibitory effect of the antisense oligonucleotides (Z-1) on EBV replication was tested as a function of the time of antisense oligonucleotide addition (Fig. 6). Cultures of Akata cells received the PS- or PO-antisense oligonucleotide (i) 3 h before, (ii) simultaneously with, or (iii) 3 h after anti-IgG stimulation. Synthesis of linear EBV DNA was monitored by Gardella gel analysis 24 h after anti-IgG stimulation. Addition of the PS-antisense oligonucleotide at 0 h and 3 h after anti-IgG stimulation resulted in a markedly reduced inhibition of EBV DNA replication compared with addition of the antisense oligonucleotide 3 h before anti-IgG stimulation. In the cells treated with the PO-antisense oligonucleotide at 0 h and 3 h after anti-IgG stimulation, there was no inhibitory effect.

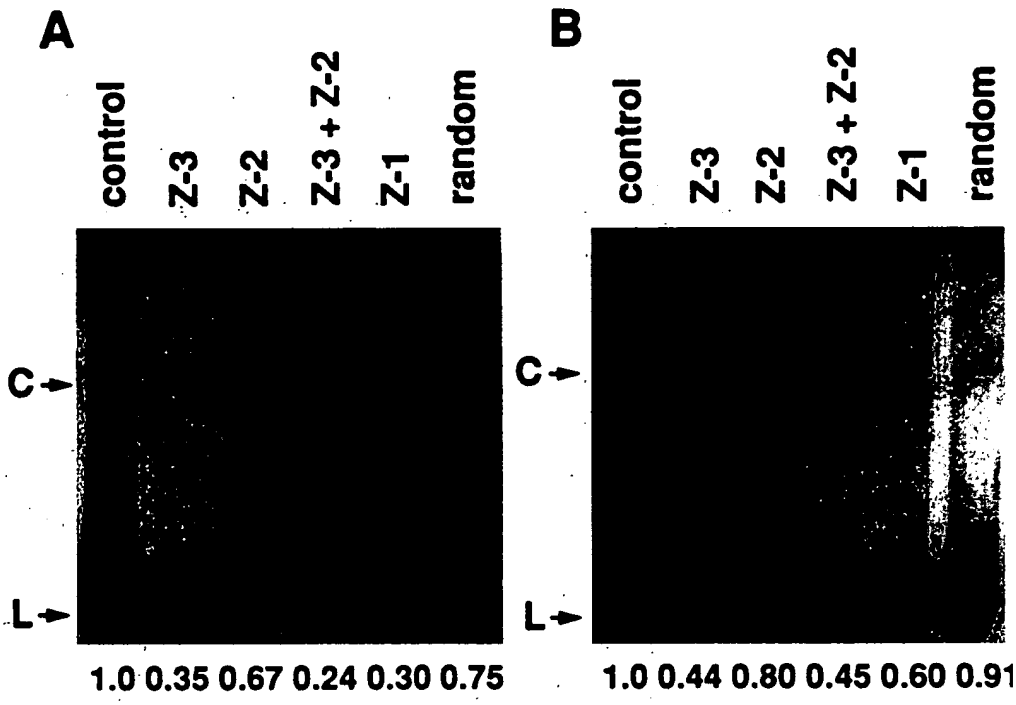

Fig. 7. Comparison of the amounts of linear EBV DNA in Akata cells after treatment with antisense oligonucleotides with three different sequences. Akata cells were preincubated for 3 h with the various anti-BZLF1 antisense oligonucleotides (Z-1, Z-2 or Z-3) singly or in a combination of Z-2 and Z-3 (each in the same concentration as in the experiments with a single oligonucleotide), or with the random oligonucleotides, stimulated with anti-IgG for 24 h, and the amounts of linear EBV DNA were assayed by Gardella gel analysis. The amount of linear DNA relative to that from anti-Ig-stimulated cells without the oligonucleotide treatment (control) is shown below each lane as described in the legend to Fig. 4. (A) PS-oligonucleotides (12.5 μM); (B) PO-oligonucleotides (25 μM). C, circular EBV DNA; L, linear EBV DNA.

3.4. Antisense oligonucleotide inhibition of the induction of EBV replication depends upon the sequences of the oligonucleotides

In addition to the active Z-1 antisense oligonucleotides, we examined two other oligonucleotides, designated Z-2 and Z-3, for their ability to inhibit productive EBV replication. Z-2 and Z-3 are adjacent 20-mer oligonucleotides which partially overlap Z-1, but extend beyond the Z-1 sequence in the 5′ untranslated region and in the open reading frame, respectively (Fig. 1). As shown in Fig. 7, both the Z-3 PO- and PS-oligonucleotides suppressed the synthesis of linear EBV DNA to levels comparable with those with the Z-1 oligonucleotides. On the other hand, treatment with the Z-2 oligonucleotides resulted in only a modest inhibition of linear DNA synthesis. Addition of the Z-2 oligonucleotides to the Z-3 oligonucleotides did not significantly enhance the level of inhibition observed in cells treated with the Z-3 oligonucleotides alone. These results were reproducible in two independent experiments. Similar results were observed at the level of ZEBRA synthesis (Fig. 8). The degrees of inhibition of ZEBRA synthesis and of EBV productive replication were very comparable.

3.5. Antisense oligonucleotides suppress EBV replication in the EBV producer cell line P3HR-1

Once we had shown that anti-BZLF1 antisense oligonucleotides could inhibit de novo EBV replication, we wanted to study their effect on an

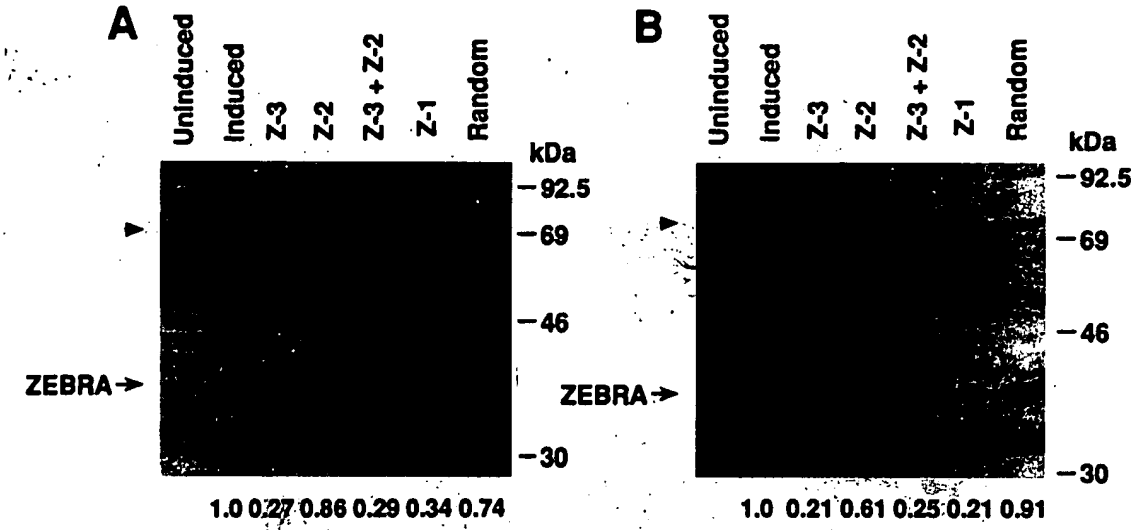

Fig. 8. Immunoblot analysis of ZEBRA induction in Akata cells treated with antisense oligonucleotides with three different sequences. Akata cells treated with the various anti-BZLF1 antisense oligonucleotides (Z-1, Z-2 or Z-3) or with the random oligonucleotides (as described in the legend to Fig. 7) were stimulated with anti-IgG, and immunoblot analysis was processed. Two control samples without the oligonucleotide treatment are included (induced, anti-Ig-stimulated cells; uninduced, unstimulated cells). The relative amount of ZEBRA expression is shown below each lane as described in the legend to Fig. 2. The levels of 72-kDa protein (arrowhead) are independent of the oligonucleotide treatment. (A) PS-oligonucleotides (12.5 μM). (B) PO-oligonucleotides (25 μM).

ongoing viral replication. For this purpose, the virus producer cell line P3HR-1 was used. Cultures of P3HR-1 cells were treated with PS-antisense oligonucleotide Z-1 for 16 days. The cells were subcultured and replenished with the PS-oligonucleotides every second day, and samples were taken for Gardella gel analysis to examine the amounts of linear EBV DNA (Fig. 9). Antisense oligonucleotide inhibition of the replication of linear EBV DNA depended upon the dose of the oligonucleotide and duration of the incubation. The PS-random oligonucleotides also affected the amounts of EBV DNA, especially at the higher concentrations (12.5 μM) and after longer cultivation (10–16 days). However, the inhibition caused by the anti-BZLF1 antisense oligonucleotide was greater than by the random oligonucleotides. Cell viability was not affected throughout the culture period as judged by exclusion of trypan blue dye (data not shown).

In parallel with the assay of linear EBV DNA, the expression of EBV antigens in P3HR-1 cells treated with the PS-antisense oligonucleotide (2.5 μM) or with the PS-random oligonucleotides (2.5 μM) was assessed by immunofluorescence (Fig. 10). About 10% of the untreated cells expressed EA-D and VCA. After only 2 days, there was no inhibitory effect on the expression of these antigens. Prolonged treatment, up to 16 days, both with the PS-antisense oligonucleotide and with the PS-random oligonucleotides resulted in a reduction in number of VCA-positive cells. However, the reduction by the random oligonucleotides was consistently less than the sequence-specific antisense oligonucleotide at all time points. EA-D expression was nearly unaffected by the random oligonucleotides at the early stage of cultivation, whereas the antisense oligonucleotide decreased the number of EA-D-positive cells to a level comparable with that of VCA-positive cells. These results suggest that the antisense oligonucleotide inhibited viral replication in P3HR-1 cells as well.

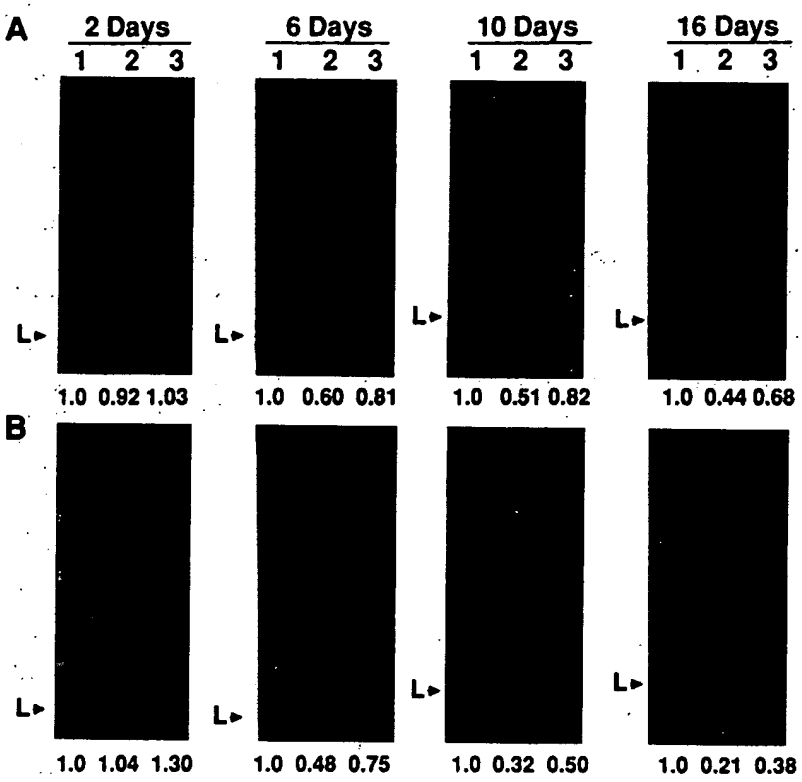

Fig. 9. EBV DNA analysis in antisense oligonucleotide-treated P3HR-1 cells. P3HR-1 cells were untreated (lanes 1), or treated with the PS-antisense oligonucleotide Z-1 (lanes 2) or with the PS-random oligonucleotides (lanes 3) at concentration of 2.5 μM (A) or 12.5 μM (B). The cells received the oligonucleotides every second day, and at several time points, samples were processed for Gardella gel analysis to examine the amounts of linear EBV DNA. The level of linear DNA is presented below each lane as relative amount. The level in untreated cells at each time point is standardized at 1.0. L, linear EBV DNA.

4. Discussion

In this study, we have demonstrated that antisense oligodeoxynucleotides (Z-1) complementary to a sequence around the AUG AUG translation initiation codons of the BZLF1 transcript led to a substantial inhibition of ZEBRA synthesis and of productive EBV replication in anti-IgG-stimulated Akata cells. Induction of the viral lytic cascade was monitored by expression of early and late EBV antigens (EA-D and VCA) and virion DNA synthesis (linear EBV DNA). Antisense oligonucleotides inhibited these parameters more strongly than random oligonucleotides. Sense and reverse sequences had the same lower inhibition of linear EBV DNA synthesis as the random oligonucleotides, showing that the antiviral effects were sequence specific. The results with the reverse sequence show that the antiviral effects were not due simply to the particular combination of nucleotides. The results with the random sequences, as well as with the sense and reverse sequences also show that it was not due to the phosphorothioate-modified backbone, as is also apparent from the results with the unmodified antisense oligonucleotides. Antisense oligonucleotides appeared to suppress the synthesis of ZEBRA selectively and they did not show a general inhibitory effect on protein synthesis, since the levels of another protein, CD 19, was not affected in the presence of the antisense oligonucleotides.

The inhibition of BZLF1 was sequence-specific since both the PO- and PS-random oligonucleotides had hardly any effect on ZEBRA synthesis. These observations suggest that the inhibition of viral replication is a result of a blockage of ZEBRA synthesis at an early stage of the viral lytic cascade. The observed partial reduction of the amounts of linear DNA by the PS-random compounds may be explained by the known inhibition of EBV DNA polymerase by PS-oligonucleotides in a non-antisense fashion (Yao et al., 1993). The observed inhibition by the PS compounds appeared to be an additive effect of the phosphorothioate and the sequence-specific antisense oligonucleotide.

Expression of BZLF1 in latently infected B cells is sufficient to activate the productive replication

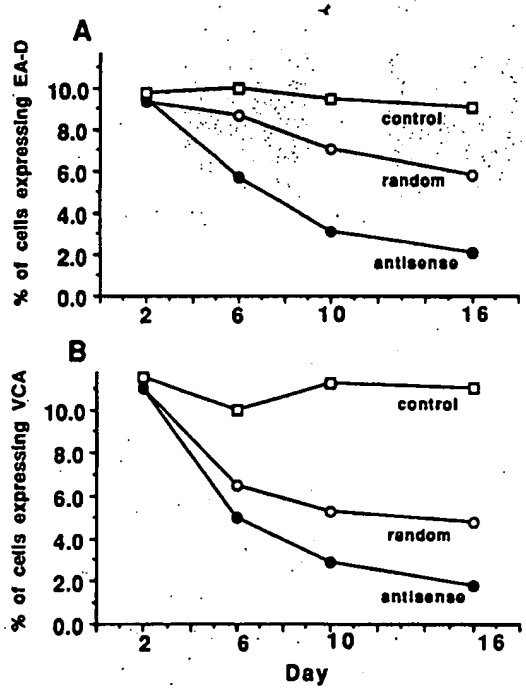

Fig. 10. Immunofluorescence analysis of EA-D and VCA expression in antisense oligonucleotide-treated P3HR-1 cells. P3HR-1 cells were incubated in the presence of the PS-antisense oligonucleotide Z-1 (2.5 μM) or of the PS-random oligonucleotides (2.5 μM), or without oligonucleotide treatment, as described in the legend to Fig. 9, and analyzed for EA-D and VCA expression. (A) EA-D expression; (B) VCA expression.

of EBV. The results shown in this paper are the first instance where it is shown that partial inhibition of this immediate early (IE) gene is sufficient to suppress the induction of the productive EBV replication. It also shows that ZEBRA is not produced in excess: 12.5 μM of the PO oligonucleotide Z-1 suppressed ZEBRA synthesis by 60% and suppressed the downstream events to comparable percentages: EA-D by 43%, VCA by 36% and linear EBV DNA synthesis by 47% (Figs. 2–4). If ZEBRA were synthesized in excess, a 60% reduction would not be expected to have any substantial effect on these downstream events.

The sequence-specific effects became clear when unmodified PO-antisense oligonucleotides were used. These PO-compounds inhibited the synthesis of EBV antigens and linear DNA, but 50% inhibition occurred at about ten times higher concentration than PS-antisense compounds. This difference is probably due to the much shorter half-life of the PO-compounds (Wickstrom, 1986; Stein and Cohen, 1988) and also to the added non-antisense inhibition of EBV DNA polymerase by PS-compounds (Yao et al., 1993). The sequence specificity was also shown when the effect of the three oligonucleotides, Z-1, Z-2 and Z-3, were compared. The Z-1 and Z-3 oligonucleotides were highly effective in inhibiting viral replication; Z-2, on the other hand, showed effects similar to the random oligonucleotides. At this stage, we can only speculate why Z-2 has so little antiviral effect.

The observed inhibition of viral DNA synthesis by PO compound may be the combined effect of suppression of early gene products needed for DNA replication as well as the lower concentration of ZEBRA available which is required for activation of ori_{Lyt} (Schepers et al., 1993).

In some instances antiviral properties of PS and PO oligonucleotides have been found to be sequence-specific although not in an antisense way (Fennewald et al., 1995). These compounds are hypothesized to affect viral absorption and/or penetration in a sequence-specific effect by an unknown mechanism (Fennewald et al., 1995). The antiviral effects described in the present study appear to be truly antisense effects: the synthesis of the gene product (ZEBRA) of the targeted gene

was inhibited, but not that of another B cell protein, CD 19.

The amounts of the circular form of EBV DNA were also reduced by treatment of the cells with the antisense oligonucleotides as compared with those seen without oligonucleotide treatment. So far, we did not investigate the reason for this reduction, but one hypothetical explanation could be that EBV DNA polymerase is involved in the synthesis of circular EBV DNA in cells following activation of viral replication, as hypothesized by Shaw (1985). In that study, the amounts of circular EBV DNA were significantly reduced in P3HR-1 cells treated with inhibitors specific for viral DNA polymerase relative to control cells without drug. It was also demonstrated that when the virus production was enhanced by stimulation of the cells with TPA the number of circular genomes increased (Shaw, 1985). In fact, our data demonstrated that the amounts of circular EBV DNA in Akata cells were enhanced about three times after anti-IgG-stimulation (Figs. 4 and 6), but they returned to the control levels of unstimulated cells when the cells were treated with high concentrations of the PS-antisense oligonucleotide. Thus, it is possible that the inhibition of induction of the viral productive cycle by antisense oligonucleotides would result in a lower amplification of the amounts of circular EBV DNA.

The specificity of the antiviral replicative effect of the anti-BZLF1 antisense oligonucleotides was further demonstrated by the decrease in inhibition when the antisense oligonucleotides were added to Akata cells at 0 h or 3 h after induction of the viral lytic cycle instead of 3 h before induction. These results indicated that antisense oligonucleotides have a low effect, once ZEBRA synthesis has started. This also suggested that preincubation may be necessary for the antisense oligonucleotides to enter the cells. It was reported that a 15-mer unmodified oligonucleotide penetrated human T-cells within 2 h in medium containing 1% fetal calf serum (Harel-Bellan et al., 1988). In the present study, the cells were pretreated for 3 h with the oligonucleotides prior to stimulation, which was sufficient to show their antiviral effects. Subsequent to the experiments shown here, it was found that this period of pre-incubation can be shortened to 1–2 h without affecting the antiviral effects (E.-M. Enzinger, J.E. Monroe and C. Mulder, to be published).

Antisense oligonucleotide inhibition of EBV replication is not limited to the induction of virus in non-producer cells. EBV replication in P3HR-1 cells was similarly inhibited by antisense oligonucleotides. In this case, it took 6 days for detectable inhibition. Yao et al. (1993) showed inhibition of EBV DNA polymerase and virion formation in a subline of P3HR-1 by several defined 28-mer PS oligomers. They showed more than 90% inhibition after 5 days incubation in 1 μM PS $(dC)_{28}$ or an anti-DNA polymerase antisense 28-mer. The differences between their results and the ones shown in this paper can probably be explained by several factors: (1) different assays, production of infectious virions vs. synthesis of linear virion DNA and EBV proteins in this paper, (2) different properties of the cell lines (e.g. 20% vs. 5% producing cells at 37°C) and (3) the very low cell concentrations used by Yao et al. (1993), 5×10^4 cells/ml (vs. 1×10^6/ml in our experiments). It is known that a low cell concentration takes up considerably more oligodeoxynucleotide than a higher cell concentration (Iwanaga and Ferriola, 1993).

Cells with an active viral replication may not require de novo ZEBRA synthesis. Thus, inhibition of BZLF1 would have no effect on these cells. The BZLF1 activation may be required only in cells newly recruited to the producer state and antisense oligonucleotides would interfere with this recruitment process. This would explain the observed long delay to attain suppression. In addition, Gardella gel analysis measures the total amounts of mature linear EBV DNA and DNA in cell-associated virions. These virions could be present in the cells for several days even if new DNA replication was inhibited, which would mask this inhibition.

In summary, the present studies show that antisense oligonucleotides against the BZLF1 transcript are capable of specifically inhibiting the synthesis of ZEBRA and subsequent productive EBV replication. Thus, inhibition of ZEBRA expression with antisense oligonucleotides represents

a novel approach for inhibition of EBV replication. The IC_{50} observed for these antisense oligonucleotides is fairly high; experiments are in progress to lower the effective dose. The selective inhibition using antisense oligonucleotides may lead to development of an effective antiviral agent for future clinical evaluation.

Acknowledgements

We are grateful to G. Miller for the gift of anti-ZEBRA antibody. We also thank J. Goodchild, D. Walther and E. Gabbidon for supplying the oligonucleotides, D. Mullen for skillful photography, and D. Celularo for secretarial help. E.-M. Enzinger was supported by a stipend from the Australian Ministry of Science and Research. This publication was made possible by a grant from Hybridon, Inc. and grant number CA-54789 from the National Institutes of Health. It's content are solely the responsibility of the authors and do not necessarily represent the official views of the US Public Health Service.

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